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13. ABSTRACT ***(Maximum 200 Words)***

This project aimed to achieve butyric acidemia, and systemic inhibition of histone deacetylase in mice. Tributyrin (butyrate triglyceride) was used as a butyrate prodrug, while methylenecyclopropaneacetate (MCPA) inhibited butyrate metabolism. We monitored physiology and blood chemistry to assure survival during acidosis. Our hypothesis was that maintenance of butyrate blood levels between 1 and 5 mM would yield regression of xenografted tumors corresponding to butyrate hypersensitive breast cancer Previous experience with organicacidemias and hypoglycin (i.e., MCPA) poisoning suggested that prolonged exposure to such acids would lead to bone marrow depression and hair loss, but would be compatible with life.

Butyric acidemia was dramatically accentuated by MCPA. However, long duration butyric acidemia was confounded by: 1) intraperitoneal tributyrin emulsion provided variable durations of butyrate release, 2) MCPA inhibition of butyrate catabolism proved limited in duration, and high plasma butyrate interfered with the ability to reattain inhibition by MCPA and 3) support of blood pH and glucose in individual animals was confounded by low blood volume (relative to instrumentation) and the extended period of critical care required. MCPA had little effect on the antiproliferative effects or deacetylase-inhibitory activity of butyrate in cultured cells. Future studies will compare continuous infusion of monobutyrin/MCPA and deacetylase-inhibitory hydroxamates.

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Butyrate therapy of poorly differentiated breast cancer

Introduction - Butyrate therapy has been considered to be both feasible and desirable, given the nature of butyrate as a natural, dietary short-chain fatty acid, based upon cell culture studies comparing the response of normal cell types (fibroblasts, gut epithelium, keratinocytes and lymphoblastoid cells) to cancer cell lines (1). The later include many of the most aggressive, poorly differentiated lines that fail to respond to genotoxic agents. These responses range from differentiation to senescence and to a more rapid, apoptotic cell death. Normal cell types and many other cancer lines cease replicating while exposed to butyrate at concentrations >2 mM, but most of the cells resume a normal cell cycle after removal of butyrate. Such activities, and the contrasting outcomes of treatment between different cell types also extend to trichostatin A (2) and more recently discovered inhibitors of histone deacetylase (3). The mediation of these actions via inhibition of this posttranslational protein modification suggests that the other cellular reactions seen during treatment with butyrate (such as the ionic perturbations caused as readily by deacetylase non-inhibitory acids as lactate and isobutyrate) should be avoidable for antineoplastic applications of agents such as butyrate.

It is clear from cell culture studies that most antitumor activities of butyrate require concentrations exceeding 2 mM and a treatment duration extending over at least 24 hours. Since butyrate is a normal energy-rich carbon source for all cells, and has a plasma halflife of less than 10 minutes, a combination of massive dosing and possibly inhibited metabolism would be required to maintain such concentrations. This rationale is supported by findings that persons with genetic deficiency of certain short chain acid dehydrogenases accumulate high micromolar concentrations of those acids in their plasma and suffer sequelae attributable to selective cytotoxicity in certain tissues (4). This project was designed to test the proposition that administration to mice of a relatively selective inhibitor of butyrate metabolism, methylenecyclopropaneacetic acid (MCPA; (5))[1] could allow exogenously administered butyrate (as the triglyceride) to accumulate for a time sufficient for a demonstration of butyrate's antitumor action. The rationale behind this proposition was that persons who ingest unripe Ackee fruit (also known as breadfruit), the richest source of the MCPA precursor hypoglycin, suffer Jamaican Vomiting Sickness, a condition that includes an extended short chain organic acidemia, bone marrow depression (pancytopenia) and hair loss (6, 7).

This project aimed to devise a strategy for maintaining butyric acidemia in mice over a 24 hour period of time, so as to maintain a nearly complete inhibition of histone deacetylase activity. The basic approach was to use butyrate triglyceride, a prodrug to butyrate much touted by the NCI (8, 9), and MCPA. We monitored both vital signs and (minimal) blood chemistry during the prolonged metabolic acidosis. Our hypothesis was that such a scheme would allow maintenance of blood butyrate concentrations at levels between 1 and 5 mM, and that each of the mice will develop hyperacetylation of chromatin core histones within 12 hours. Such hyperacetylation was

[1] Abbreviations: MCPA, methylenecyclopropylacetate; SCA, short chain organic acids: SCAD, short chain acyl-CoA dehydrogenase; IVAD, isovaleryl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; HDAC, histone deacetylase; TPN, total parenteral nutrition; SBHA, suberoylbishydroxamate; IACUC, institutional animal care and use commitee.

conjectured to be sufficient to cause extensive cytolysis in xenografted tumors whose cell cultured counterparts had known sensitivity to butyrate-induced apoptosis.

The goals of this study included:

1. Determine the pharmacokinetics of butyric acidemia, demonstrating the effectiveness of intraperitoneally-delivered butyrylglyceride in mice treated (or not) with MCPA.
2. Document core histone acetylation state of chromatin isolated from selected tissues, as an indicator of the inhibition of histone deacetylase, and thereby the bioavailability of butyrate within the target cells.
3. Document and attempt to correct untoward acute effects of MCPA and butyrate therapy. These effects were predicted to be hypothermia, hypoglycemia, metabolic acidosis, electrolyte disturbances and nausea.
4. Document untoward delayed or chronic effects of butyrate therapy, including bone marrow depression (as reflected in reductions in blood cell counts) and liver toxicity (e.g., fatty liver or necrosis).
5. Demonstrate antitumor effects of prolonged butyrate therapy, in relationship to hyperacetylation of tumor chromatin histones.

Body - As shown in fig. 1, intraperitoneal administration of tributyrin emulsion (2 g/kg) was able to provide butyric acidemia in excess of 5 mM for periods of up to one half hour in control mice, but up to 3 hr in MCPA pre-treated mice. Replicate determinations were highly variable in some experiments, especially at later times after administration of emulsion, results that were reflected in variability of the depth of depression of consciousness and stimulation of respiration[2]. We attributed this variability to unfavorable characteristics of the oil-in-water butyric triglyceride emulsion. In our hands, a glycerolized mechanical dispersion containing an FDA-approved polyethylene/polypropylene glycol surfactant (Pluronic F68) proved to have a stability toward separation over time better than lecithin, Tween or cyclodextrin-stabilized emulsions.

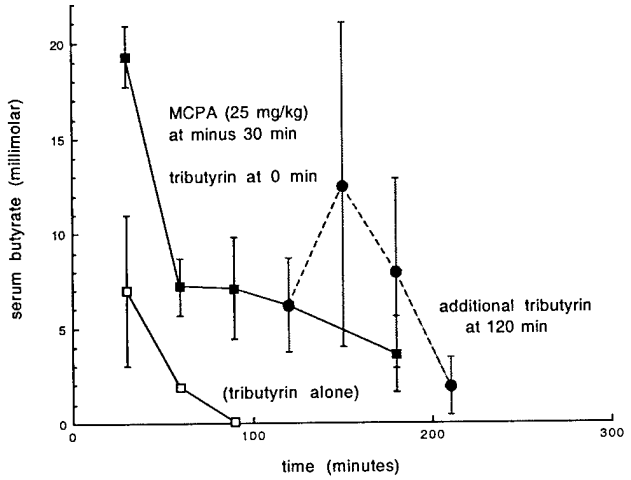

Figure 1. Effect of MCPA pretreatment on serum butyrate concentrations following a single or repeated administration of tributyrin emulsion. Mice were injected intraperitoneally with saline (open squares) or MCPA (25 mg/kg; closed squares and octagons) at 30 minutes prior to administration of a metastable tributyrin emulsion (20% in saline containing 5% glycerol and Pluronic F68 surfactant; 2 g/kg) at the start of the experiment. At 120 min, some mice received an additional dose of tributyrin. At the times indicated, mice were anaesthetized and terminally bled from the axillary artery. Serum was extracted and the organic acid fraction examined by gas chromatography. Materials eluting after a time characteristic of butyric acid were quantitated in comparison with a dilution series of butyric acid. Data are average and range for two mice each.

A second (and third) administration of tributyrin emulsion resulted in additional peak(s) of butyric acidemia (eg., fig. 1), and exacerbations of constitutional symptoms of acidosis. As expected from the variable level of the residual/background butyric acid in these mice, the results for this second peak of butyric acidemia exhibited compounded variability. However, it was nonetheless clear that the duration of this second period of butyric acidemia was abbreviated, even in MCPA-pretreated mice. This was taken to represent recovery from MCPA-induced inhibition of butyrate metabolism, presumably by resynthesis of SCAD protein, although other adaptive mechanisms, such as accelerated renal clearance of butyrate, could possibly be responsible. In agreement with the first possibility however, an additional dose of MCPA restored the prolongation

[2] Although we did not try to quantify these later effects in individual mice, consciousness appeared to be impaired in mice with blood concentrations of butyrate above ~3 mM, and respiratory stimulation increased as butyrate concentrations increased from 3 to 10 mM. Respiratory signs and the decrease in blood pH appeared to be relieved significantly by administration of bicarbonate and glucose.

of butyric acidemia from the second administration of tributyrin, although the enhancement was much less. In line with this finding of diminished activity, when MCPA (30 mg/kg) was given within an hour after tributyrin administration, the effectiveness of MCPA appeared severely blunted, and the depressed consciousness and hyperpnea of such mice appeared similar in severity and duration to mice that had received only tributyrin. This suggested to us that butyrate interfered with MCPA for uptake from the circulation, binding to SCAD, or both. (we could not reliably measure MCPA in the plasma of tributyrin-treated mice, due to its low magnitude and overlap with the huge butyrate peak on chromatograms). Use of MCPA to extend butyric acidemia over 20 hr will thus necessitate administration of this agent continuously at doses considerably higher that that which is useful in a pretreatment sense, potentially increasing the unintended inhibition of alternative enzymes such as the medium chain acylCoA dehydrogenase (10).

The modifications to the approach that suggested for extension of these studies include:

a. **Continuous infusion via intraperitoneal catheter** – We received IACUC approval for this modification to our approach. The committee insisted that we obtain swivel sets to be mounted on a collar of each animal for infusion irrespective of activity level. These devices allow freedom of movement for the animal during continuous administration of fluid. We wish to be able to modify and ascertain the infusion rates, at times increasing or decreasing the flow through one of the two lines that will converge prior to the swivel. The catheter (we have chosen a Microline crosslinked-EVA tubing, both for biocompatibility and chemical compatibility reasons) will run under the skin from the dorsal neck to the lateral peritoneum, where it will be left indwelling through the peritoneum. The wall of the catheter is cut to an open-end conical form, and held around a 22g hypodermic needle for insertion. This plastic is otherwise very soft, similar to silicone, and unlikely to puncture any hollow viscus. We have successfully tested this on a series of metaphane-anaesthetized mice. We have obtained two 10 channel syringe pumps, one for a monobutyrin and MCPA solution (the output of which will be monitored and recorded on a computer interface over time), and another, for a glucose/sodium bicarbonate solution, which is modified to allow transitory increases of individual syringes. The later will be provided for hydration and relief of untoward effects of the SCFA therapy. These two lines will be joined at the swivel by means of high pressure fittings.

b. **Monobutyrin as an alternative prodrug** – Due to its water insolubility, tributyrin has limited usefulness as a parenteral agent or as a reliable slow-release reservoir of butyrate anion. A better approach to modeling cell culture experiments may thus be parenteral infusion of a water-soluble ester or salt. The use of the sodium salt would, we predict, risk hypernatremia, especially as sodium bicarbonate will likely be needed throughout the infusion. Monobutyrin has been given to dogs and rats at 1-4 g/kg/day (isocaloric replacement for glucose), providing weight gain and no reported untoward effects (11), and it was under investigation as an energy source as a carbon/energy source in a TPN formulation for human use.

   The simplest approach to preparation of monobutyrin is partial alkaline saponification of tributyrin followed by vacuum distillation. Other workers have prepared monobutyrin by purchasing technical grade monobutyrin from Eastman Chemicals (the agent has a short shelflife) and distilling it themselves. Eastman Chemicals (now Fisher Scientific) no longer markets monobutyrin, leaving no alternative source. However, the need for vacuum distillation, and the analytical procedures required to verify its purity make it worthwhile to synthesize the agent in the laboratory (such as by simple esterification from butyryl chloride and anhydrous glycerol).

c. **MCPA as a continuous infusion as well as bolus pretreatment** – The short effective period of MCPA in treated mice indicates that mice will require continuous infusions of MCPA after the bolus pretreatment, and that the interference of circulating butyrate with supplemental MCPA will necessitate detailed testing of monobutyrin/MCPA infusion conditions. Our work has demonstrated that single dose MCPA, with or without delayed addition of

otherwise tolerated doses of tributyrin emulsion, is well tolerated even at doses 3 times that of our standard 15 mg/kg pretreatment dose. Earlier work with hypoglycin, the amino acid precursor of MCPA[3], demonstrated hypoglycemia, hypothermia, acidosis and delayed effects such as thymic involution and liver damage. Since we control for hypothermia (which has been shown to exacerbate the hypoglycemia - (12)), and ameliorate the acidosis somewhat using bicarbonate, our mice are somewhat better supported than mice in earlier, toxicology experiments. However, we cannot predict the outcome of experiments with continuous infusion with the necessarily higher doses of MCPA, especially since butyrate may selectively protect SCAD from MCPA inactivation. If MCPA now has a disproportionate inhibitory action on MCAD, since MCAD deficiency tends to be more clinically significant than SCAD deficiency , we might find that additional sequelae arise with prolonged infusion.

d. **Environmental control** – Because of the propensity of MCPA-treated mice to develop hypothermia, we have constructed a temperature controlled (33-36°C across the platform) ventilated multichambered incubator for long-term infusion, physiological monitoring and blood sampling of mice. The reproducibility and stability of chamber temperatures, and rectal temperatures of the mice, indicate that device is effective.

e. **Rapid, low sample volume analysis of blood pH and butyrate** - Our work documenting pH changes during butyric acidemia and after administration of sodium bicarbonate to such mice pointed out a deficiency in the ability to gauge the effects of the test agents and remedial interventions on individual mice. Because our pH and pCO_2 apparatus had not been miniaturized for samples under 50-100 µl, we could not monitor the condition of the mice without killing them (or observing them for respiratory activity). We have designed and begun construction of a device to measure pH and pCO_2 on smaller samples (30-50 µl), conceivably allowing serial sampling of living mice, i.e., from tail vein or intraorbital venous plexus. After reading the electrode responses, the diluted blood can be deproteinized and used for determination of glucose.

f. **Physiological monitoring** – Besides the need for blood chemistry determinations during the course of infusion, we believe that the mice would be better supported during prolonged MCPA/butyrate infusion if monitored for changes in respiratory activity, conscious movement and core body temperature. We have obtained a MacLab analog/digital converter, biopreamp and several transducers (including a bellyband for diaphramatic activity related to respiration depth and rate). The rapid response of respiratory activity to blood pH, pCO_2 and organic acids may allow us to better gauge and record the response to bicarbonate and changes in butyrate infusion rate, while core body temperature may provide an indication of the severity of hypoglyemia.

g. **Core histone acetylation ratio** - Most phenomena associated with growth inhibition or apoptosis induction require >12-16 hr of treatment with millimolar concentrations of butyrate to be evident or significantly increased. This corresponds to the kinetics and dose response relationships of cells to butyrate as evident in hyperacetylation of bulk histones, i.e., the species of histones H2a, 2b, 3 and 4 that are evident on Coomassie blue-stained electropherograms. Such changes are rapidly (15 min) reversed upon reduction of butyrate concentrations (2). Because the problems mentioned above have precluded maintenance of millimolar butyrate concentrations for beyond 2-3 hr, we could not test this in mice. On the other hand, cell cultures tend to be useful for answering many of these questions. We therefore performed a series of experiments to test whether MCPA would synergize with butyrate

[3] Hypoglycin is the toxic constituent present in Ackee nut, responsible for Jamaican vomiting sickness which befalls persons who ingest unripe breadfruit (Ackee nuts). Upon deamination, hypoglycin yields MCPA, which as a CoA ester acts as a suicide inhibitor of dehydrogenases SCAD (short chain acylCoA dehydrogenase), MCAD (medium chain), IVAD (isovalerylCoA dehydrogenase). Because of the general depression in fat metabolism, both from inhibition of dehydrogenases acting upon acyl groups less than 10-12 carbons, and depletion of CoA, general energy metabolism becomes deficient. This leads to excessive, life-threatening consumption of glucose, and hypothermia.

concentrations near 100 micromolar (histone deacetylase is half inhibited at 150 μM,) in effecting hyperacetylation of histones. I therefore treated two prostate cancer cell lines (JCA-1 and DU-145, as we are funded to do such studies of butyrate response by prostate cancer cells) with butyrate at 0, 0.03, 0.1, 0.3, 1, 3 and 10 mM butyrate, ±4 mM MCPA. MCPA caused only minor morphological effects on the cultured cells in contrast to 3 or 10 mM butyrate. It also had little effect on the cells' response to lower butyrate concentrations. Chromatin was harvested from each treatment group at 14, 24 and 34 hours and analyzed for histone electrophoretic ability (a measure of charge, which is in turn a quantum reflection of discrete acetylations). The results showed acetylation ratios to change little between 14 and 34 hr, which we believe indicates that butyrate was not consumed appreciably by the prostate cancer cell lines, in contrast to our findings with colorectal cancer cell lines. To our knowledge, the impact of intracellular metabolism of butyrate on butyrate responsiveness has not been examined since early work by Charlotte Friend (13). The prostate cancer cell lines each exhibited hyperacetylation during exposure to butyrate concentrations as low as 0.3 mM, close to the Ki for butyrate as an inhibitor of HDAC in vitro (1). The lack of significant hyperacetylation response to MCPA was however consistent with the low activity of carboxylic acids of greater than 5 carbons (2). However, a proper test of the relative activity of MCPA would require purification of the MCPA (it seems to have accumulated a polymeric degradation product with storage, even at -20°C), a difficult task given the small quantity (~100 μl) available at this time. In conclusion, however, we cannot expect that MCPA treatment will relieve the requirement for millimolar conentrations of butyrate in plasma for effective antitumor therapy, leaving us to grapple with the complications of long term metabolic acidosis.

Research accomplishments

1. Estimation of a minimally effective dose of MCPA for prolonging butyric acidemia. (We could not determine a maximally tolerated dose, as we were limited in supply and because the tolerance for tributyrin was the limiting factor in our experiments.)
2. Demonstration that MCPA-inhibitable processes, most likely beta oxidation, was the metabolic pathway that most strongly limited the duration of butyric acidemia. Other candidate metabolic processes were gamma carbon oxidation (leading to ethylmalonic acid, the principal excretion pathway in humans), and glycine conjugation (a major pathway for excretion of organic acids in rodents, as well as humans). Anabolic pathways, such as fatty acid synthesis from butyryl-CoA are also likely to be minor pathways, although many (such as fatty liver) would likely have proven problematic during extended therapy.
3. Demonstration that butyric acidemia exceeding 20 mM was acutely tolerable, and that mice could recover consciousness after 3-4 hours of butyric acidemia, especially when bicarbonate, glucose and a warm environment was provided. The few deaths that we did observe, however inexplicable, were related to use of freshly-prepared tributyrin emulsion.
4. Demonstration that butyrate competes with MCPA for the ability of the later agent to inhibit SCAD in vivo. While this had the practical detriment of limiting our ability to maintain butyric acidemia, a practical benefit of this finding is that butyrate could be used prophylactically to block the effects of exposure to an MCPA-type agent. Whether this interaction could be exploited to any practical end remains to be shown. However, we have considered an offshoot of the phenomenon to be the blocking of MCPA inhibition of MCAD (10)using octanoate or spiropentane acetic acid (16).

Reportable outcomes

We believe that this work would be of insufficient consistency to justify publication. The variability of the pharmacokinetics after administration of tributyrin emulsion leaves doubts as to the desirability of using this approach.

Because we believe that an agent like SBHA (or one of the proprietary/natural product HDAC inhibitors) would excel for antitumor trials, we will likely continue the work with butyrylmonoglyceride (and stabilized derivatives) in parallel with a study of SBHA. There remains a much richer literature relating to the effects of butyrate on cultured cells, as compared to any other HDAC inhibitor, so a simple comparison of butyrate with any given agent lends confidence that the earlier work is similarly relevant.

We have considered writing up a quick note to alert other scientists about the problems with tributyrin, as far too many papers are published which praise this agent as if it were a practical solution, thinking only about problems with high doses of butyrate salt counterion. There may however be no forum for presentation of such 'negative' results, and we will instead strive to present our criticisms in connection with a demonstration of the activity of medium chain hydroxamates..

Conclusion and prospects

This project evolved into one which focussed increasingly on butyrate as a determinant of life-threatening acidosis, on miniaturization of procedures for continuous infusion and physiological monitoring of mice, and of dealing with pharmaceutically difficult agents. It may also be in hindsight obvious that butyrate would inhibit the action of MCPA as a competitive inhibitor of initial binding to the SCAD. Since few instances of such drug interaction are well studied, a study of this interaction in mice might have been a valuable contribution to the literature, and might have been pursued had it not been for a lack of resources and the other complications described herein. We are now equipped to perform such a study, but fear that we would need to enlist (at great expense) a collaborator with expertise in acute critical care and respiratory/metabolic complications of organic acidemias. We believe that one or more of the >6 fungal HDAC inhibitors, or one of the synthetic inhibitors such as SBHA (3) will prove to be far better tolerated, more easily administered, less likely to have complicated pharmacokinetics and more likely to be developed by the pharmaceutical industry.

Despite the advances in molecular understanding of the functions of nuclear protein acetylation in gene expression, and the identification of uniquely synergistic combinations of HDAC inhibitors and other agents, there appears to be a minimum effective duration of HDAC inhibition for durable antiproliferative action lasting typically between 12-48 hours if we are to achieve effective antitumor activity. At present it appears that the lower limit of this time correlates with maximal hyperacetylation, just as the inhibition of cell growth correlated with accumulation of tetraacetylated histone H4 (14, 15). The period of time needed for maximal loss of replicative abilities by susceptible cells is less well understood, but correlates somewhat with cell cycle-related factors. Because MCPA-poisoned or SCAD/IVAD-deficient persons suffer hair loss and bone marrow depression after a significant period of organic acidemia, we continue to believe that HDAC inhibitory SCA's will have antitumor effects if maintained as continuous dosing for 1-2 days, if this were tolerable. We would prefer to use an agent such as SBHA, analogs of which have been shown to be tolerated at the concentrations needed to effect antitumor actions *in vivo* (3). This agent (SBHA) is approximately 100 fold more potent than butyrate on a weight basis with respect to both histone hyperacetylation and cell death induction in cultured prostate cancer cell lines (manuscript in preparation).

Our attempts at butyrate therapy using MCPA and tributyrin highlighted several problems that ranged from correctable (use of infusion of a water soluble monoglyceride rather than bolus injections of the intractable oil-in-water emulsions of tributyrin) to unavoidably problematic (loss of consciousness and acidosis at millimolar plasma concentrations of butyrate). There is no reason to expect that the coma that accompanies millimolar butyrate therapy will be either surmountable or preventable. While significant enhancements of hemoglobin F expression can be achieved after bolus administration of butyrate to children with hemoglobinopathies , antitumor actions described to date appear to trace to mechanisms other than incremental increases in gene expression. It remains to be shown whether butyric acidemia could be maintained within the

potentially tolerable range of 1-5 mM, where neither overwhelming acidosis or restored deacetylase action would confound a successful outcome.

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